5 NORMALIZATION WIZARD AND AMPLIFICATION SET UP

The Normalization Wizard is designed to utilize previously generated DNA quantitation data (ng/µL) to create a customized BioMek® 2000 Automation Workstation method for the dilution of those samples to a concentration specified by the user. In particular, an Excel table containing the Aluquant™ Human DNA Quantitation System data is imported into the Normalization Wizard as part of the procedure for the generation of a customized robotic method for diluting the samples on the BioMek® 2000 Auotmation Workstation. An aliquot of the diluted DNA samples, programmed in by the user, is then added to PowerPlex® 16 BIO amplification master mix, which is aliquoted by the robot into PCR amplification tubes. When the entire process is complete, a set of PCR reactions will be prepared in a customized fashion and ready for amplification.

5.1 EQUIPMENT

5.1.1 BioMek® 2000 Automation Workstation

5.1.2 BioMek® MP200 Pipette Tool

5.1.3 BioMek® MP20 Pipette Tool

5.1.4 BioMek® P200L Pipette Tool

5.1.5 BioMek® P20 Pipette Tool

5.2 MATERIALS

5.2.1 2 - Black PCR support bases (96 well) – ABI Cat# N801-0531

5.2.2 MicroAmp® tubes (attached caps) – USA Scientific # 1402-8100 (clear) or #1402-8108 (multi- colored)

5.2.3 Modular reservoir full module 150mL - Beckman Catalog # 372784

5.2.4 96 well normalization plate – Innovative Microplate Catalog # S30026

5.2.5 4 - Beckman 24 Microfuge tube holders- Beckman Catalog # 373661

5.2.6 Beckman white 1.5 mL tube inserts- Beckman Catalog # 373656

5.2.7 P250 Tips – no aerosol barrier - Beckman Catalog # 372655

5.2.8 P250 Tips - aerosol resistant – Beckman Catalog # 140505

5.2.9 P20 Tips - aerosol resistant – Beckman Catalog # 609043

5.2.10 Microcentrifuge tubes, 1.5 mL with unattached lids

5.3 REAGENTS

5.3.1 Type I Sterile Water

5.3.2 AmpliTaq™ Gold DNA polymerase

5.3.3 Powerplex® 16 BIO System amplification kit

5.4 PREPARATION FOR USING THE NORMALIZATION WIZARD

NOTE: The tools and the BioMek® robot must be wiped off with 10% bleach, followed by Isopropyl alcohol or Ethanol prior to running the Normalization Wizard method.

5.4.1 Open the AluQuant® v3.0 (AQ3.0) file (e.g., DATE, OPERATOR's Initials, Run Time – AM or PM, if necessary robot initial) located on the "C" drive, containing the concentration data for the plate of DNA extracts of interest that will be prepared for amplification set up (Figure 1). At the bottom of the AluQuant® calculator spreadsheet select the BioMek® tab to open the import data screen.

NOTE: If this transfer step has already been performed, proceed to step 5.4.6.

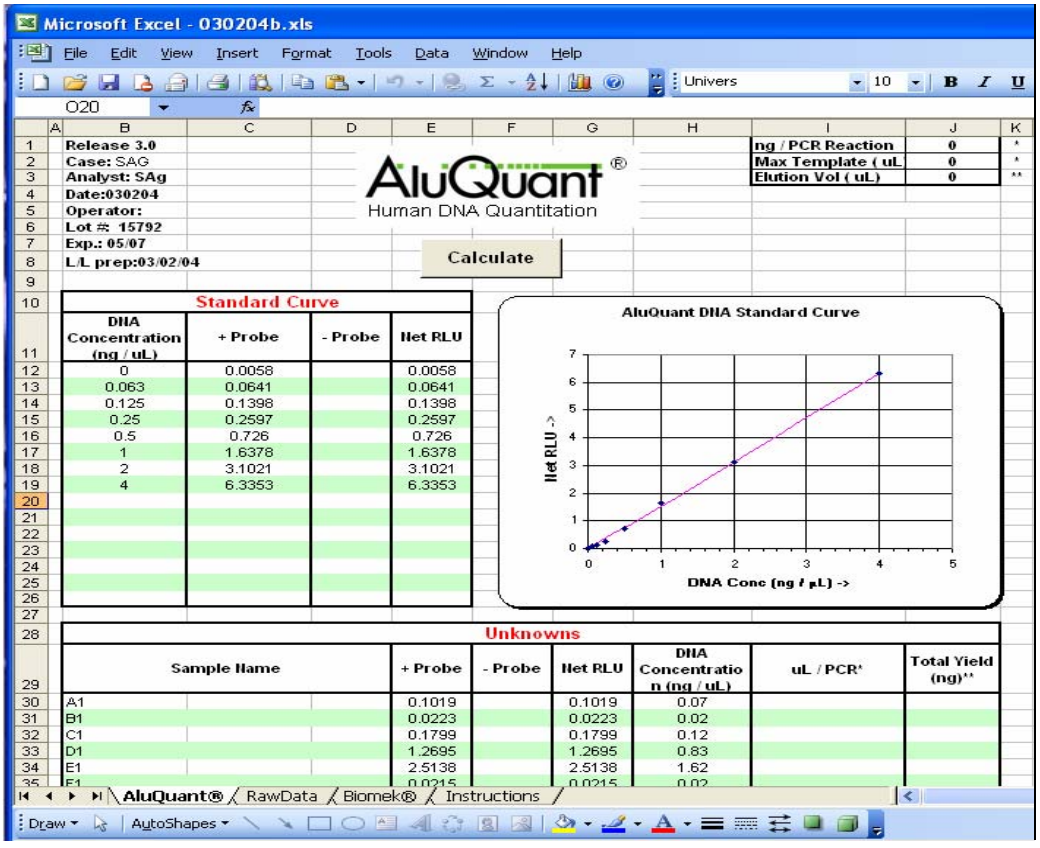


Figure 1. AluQuant® v3.0 data file

5.4.2 Transfer the quantitation data into the BioMek® Import Data spreadsheet by clicking on the "Import Data From AluQuant® Sheet" button on the screen (Figure 2).

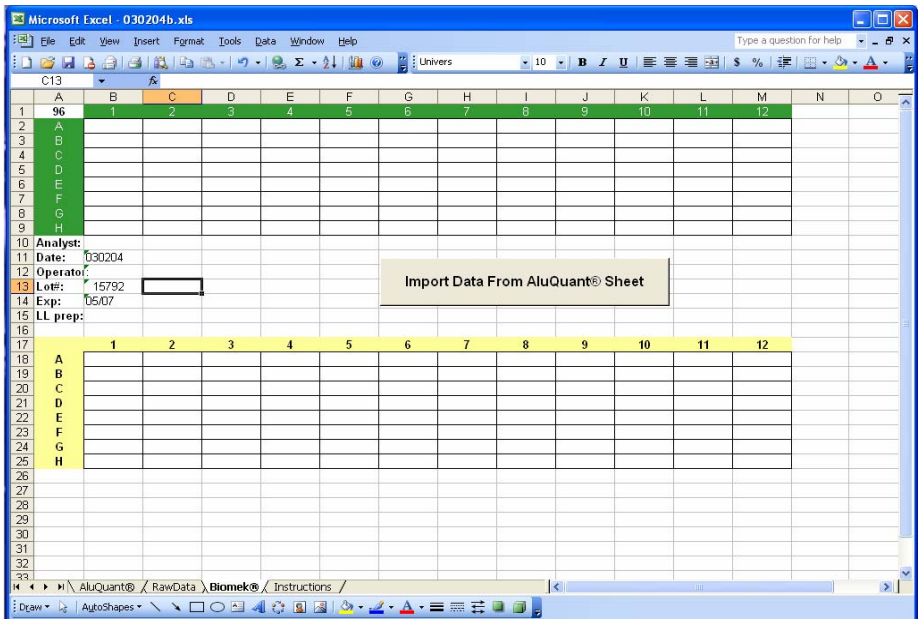


Figure 2. BioMek® Import Data Spreadsheet

5.4.3 When the window pops up prompting the user how the data should be transferred (Figure 3); select Full Plate (Wells A1→H11), then click OK.

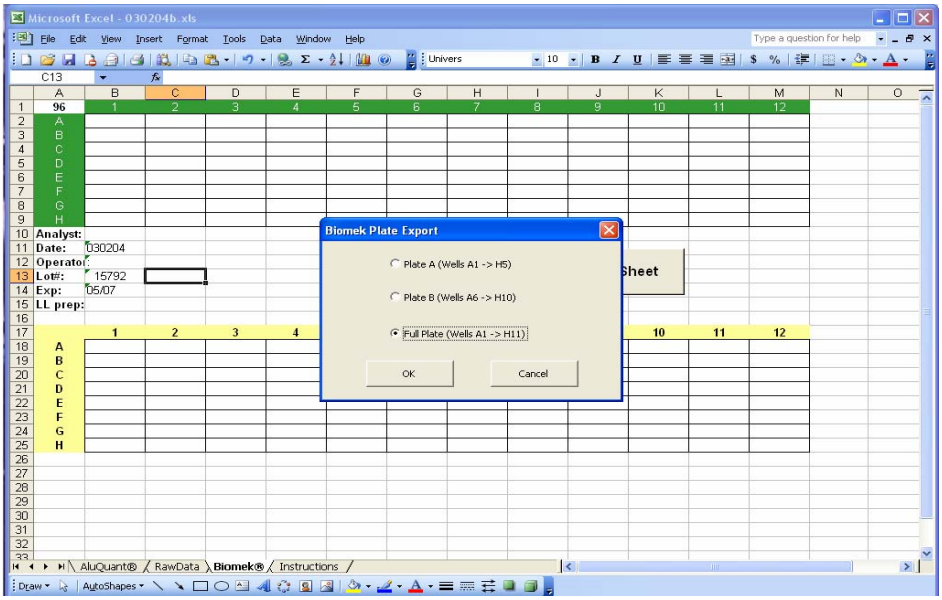
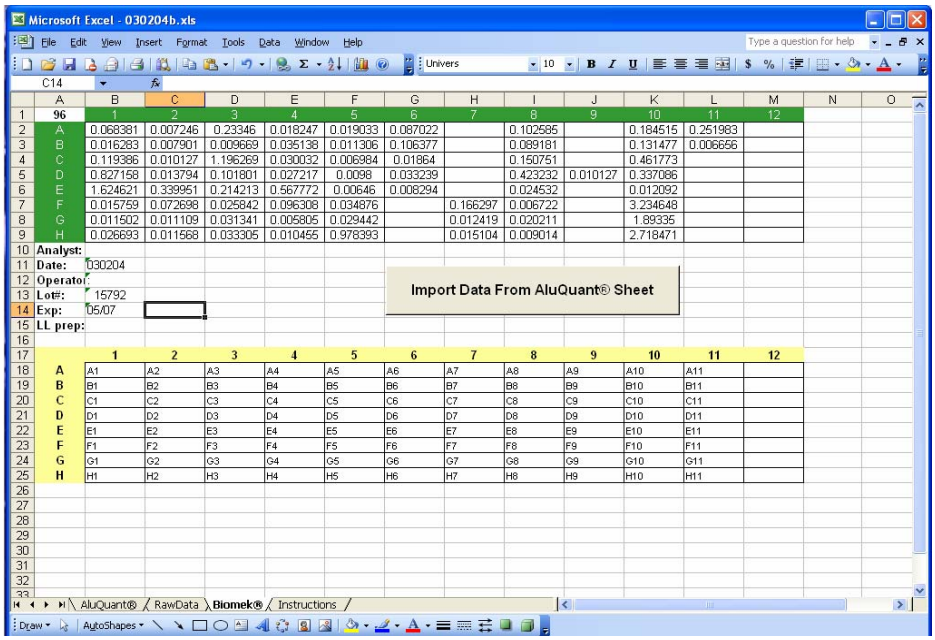


Figure 3. BioMek® Plate Import Prompt

5.4.4 The quantitation data will transfer to the 96 well format in the BioMek® Import Data spreadsheet. This format is recognizable by the Normalization Wizard software method for customized DNA sample dilution (Figure 4).



96	1	2	3	4	5	6	7	8	9	10	11	12
A	0.068381	0.007246	0.23346	0.018247	0.019033	0.087022		0.102585		0.184515	0.251983	
B	0.016283	0.007901	0.009669	0.035138	0.011306	0.106377		0.089181		0.131477	0.006656	
C	0.119386	0.010127	1.196269	0.030032	0.006984	0.01864		0.150751		0.461773		
D	0.827158	0.013794	0.101801	0.027217	0.0098	0.033239		0.423232	0.010127	0.337086		
E	1.624621	0.339951	0.214213	0.567772	0.00646	0.008294		0.024532		0.012092		
F	0.015759	0.072698	0.025842	0.096308	0.034876		0.166297	0.006722		3.234648		
G	0.011502	0.011109	0.031341	0.005805	0.029442		0.012419	0.020211		1.89335		
H	0.026693	0.011568	0.033305	0.010455	0.978393		0.015104	0.009014		2.718471		

Analyst:
Date: 030204
Operator:
Lot#: 15792
Exp: 05/07
LL prep:

	1	2	3	4	5	6	7	8	9	10	11	12
A	A1	A2	A3	A4	A5	A6	A7	A8	A9	A10	A11	
B	B1	B2	B3	B4	B5	B6	B7	B8	B9	B10	B11	
C	C1	C2	C3	C4	C5	C6	C7	C8	C9	C10	C11	
D	D1	D2	D3	D4	D5	D6	D7	D8	D9	D10	D11	
E	E1	E2	E3	E4	E5	E6	E7	E8	E9	E10	E11	
F	F1	F2	F3	F4	F5	F6	F7	F8	F9	F10	F11	
G	G1	G2	G3	G4	G5	G6	G7	G8	G9	G10	G11	
H	H1	H2	H3	H4	H5	H6	H7	H8	H9	H10	H11	

Figure 4. Quantitation data transferred to the BioMek® Import Data spreadsheet.

5.4.5 Once the quantitation data of the AluQuant® file (sample file name as in step 5.4.1) has been imported to the BioMek® sheet, the file must be saved to a designated location in the computer workstation using the appropriate file name. The Normalization Wizard simply needs the AluQuant file name for the appropriate sample set for direct import of the data into the Normalization program. The AluQuant Calculator software can now be closed.

5.4.6 Open the BioWorks folder on the desktop. Click on the Lab Book Manager icon.

5.4.7 A list of the Lab Books will be displayed as shown in Figure 5. Highlight the Wizard Lab Book and click the "Set as Current Lab Book" button. Close out the Lab Book Manager window.

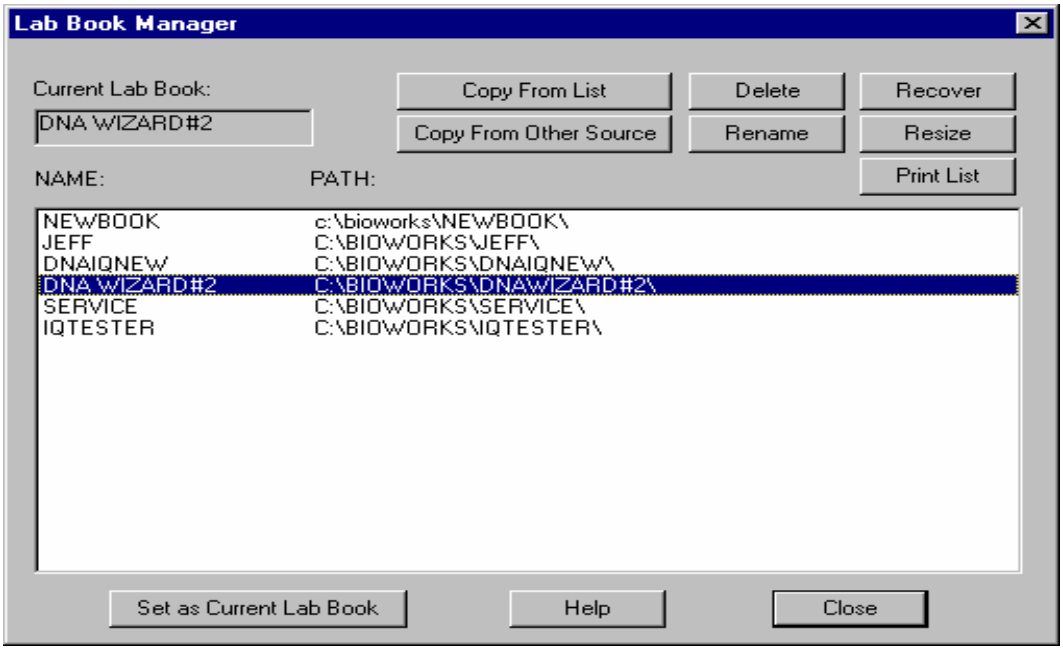


Figure 5. Lab Book Manager

5.4.7.1 If a Wizard Lab Book does not exist, create one by double-clicking on the Lab Book Manager ion in the Bioworks folder. Click on the button "Copy from List". Any of the Lab Books can be used, but it is probably best to select the DNA IQ or DNA IQ New Lab Book.

5.4.8 A prompt will ask for a name for the lab book. Name it Wizard and provide the path for the new Lab Book: C:\BIOWORKS\"WIZARD".

5.5 CREATING A NORMALIZATION WIZARD METHOD

5.5.1 In the BioWorks folder, double click on the Normalization GI Wizard icon to initiate the program. The first box is the introduction, select 'Next' to begin generating the normalization plate protocol.

5.5.2 The next dialog box to appear will be the Import Plates screen (Figure 6). Click on the Import Plates button and select the AluQuant® v3.0 file that contains the quantitation data for the samples that will be set up for STR amplification (eg 030204sag). The Import Plates box will have "BioMek®" listed under the Plate Name, the AluQuant® run filename will not be displayed. The lowest and the highest sample concentrations present in the opened file will be shown; select "Next" to proceed. NOTE: if a sample has a concentration greater than 17.0 ng/µL, that sample should be excluded from the process and the examiner notified that the sample must be manually prepared for amplification.

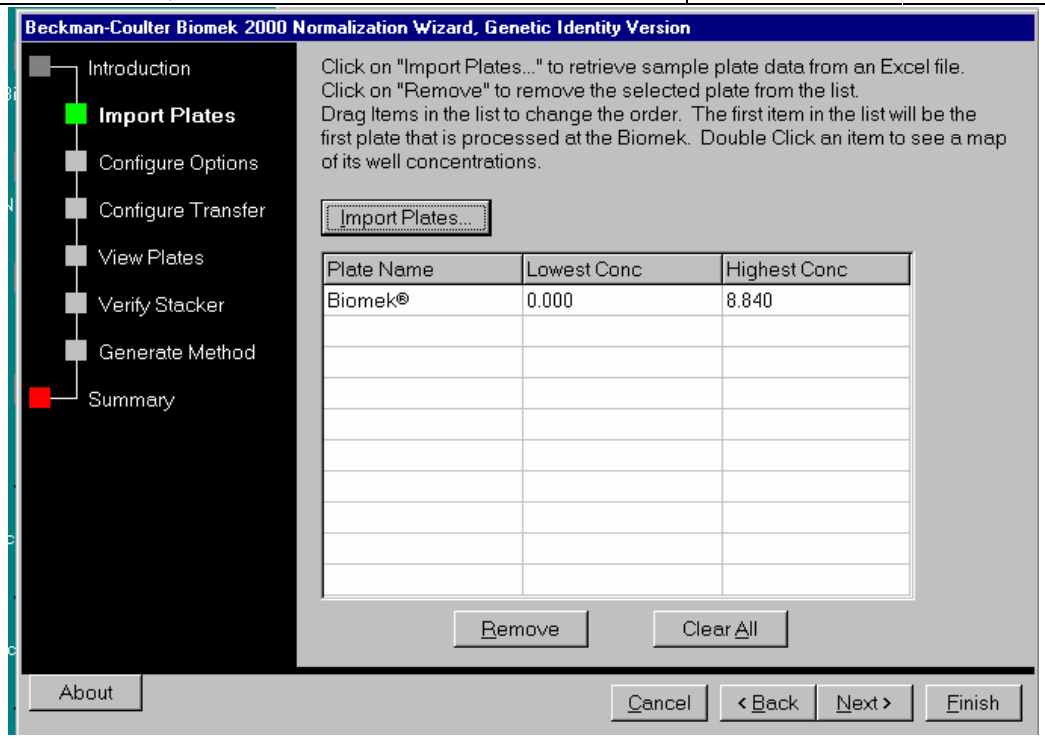


Figure 6. Importing plates into the Normalization Wizard.

5.5.3 Change the Final Concentration and the Sample Volume from a target of 0.15 to 0.20 ng/µL for the concentration field and 8 to 10 µL for the volume field as shown in the boxes below on the Configure Options dialog screen (Figure 7); select "Next" to proceed.

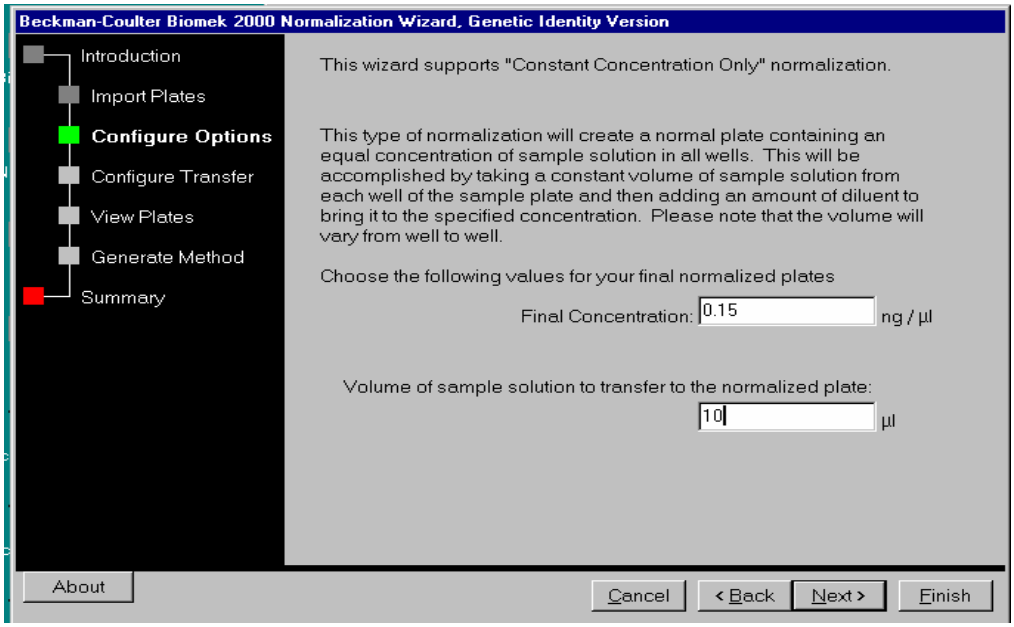


Figure 7. Settings for Final DNA Concentration and the Sample Volume.

5.5.4 Verify that the parameters are set as appears in the boxes below on the Configure Transfer screen (Figure 8):

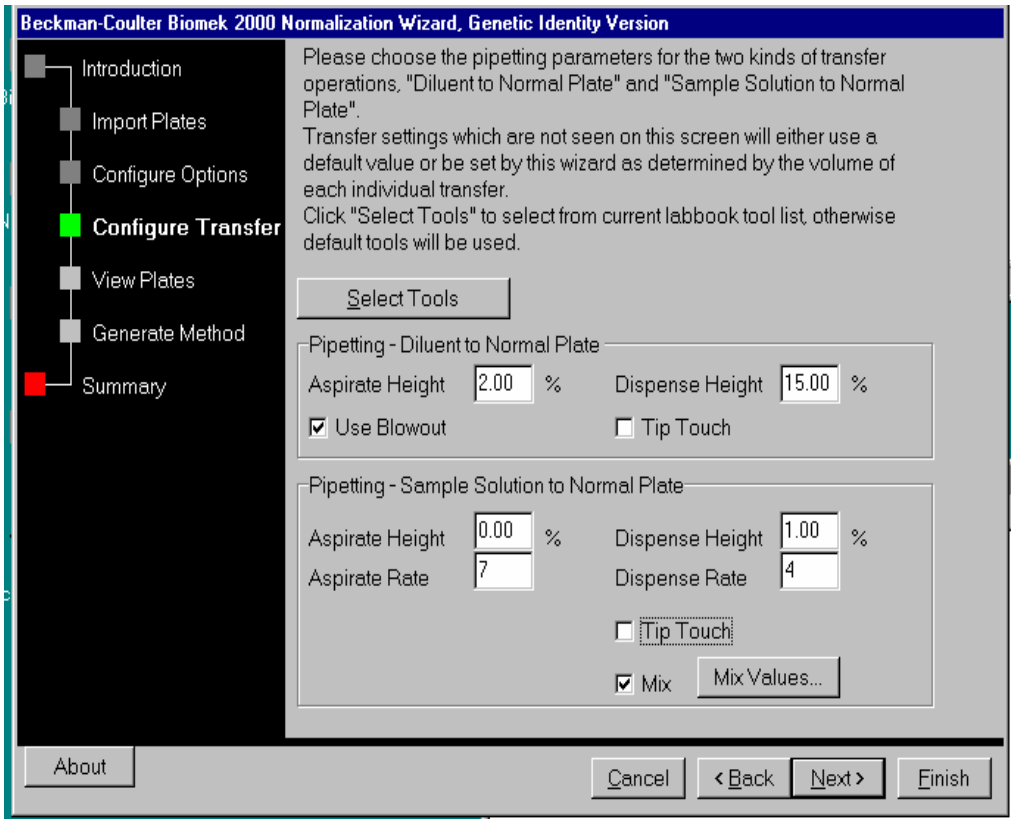


Figure 8. Setting pipetting parameters.

5.5.5 Uncheck the "Tip Touch" box on the Configure Transfer screen. Check the "Mix" option box then click on the "Mix Values" button and change the settings to the values seen on the Mix Values screen below (Figure 9). Select OK, then "Next" on the Configure Transfer screen to proceed.

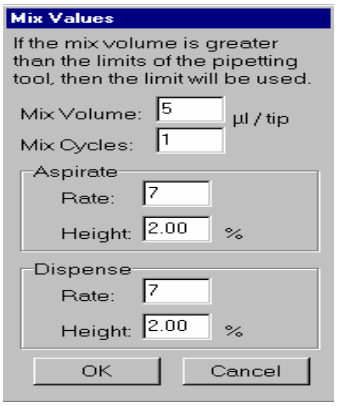
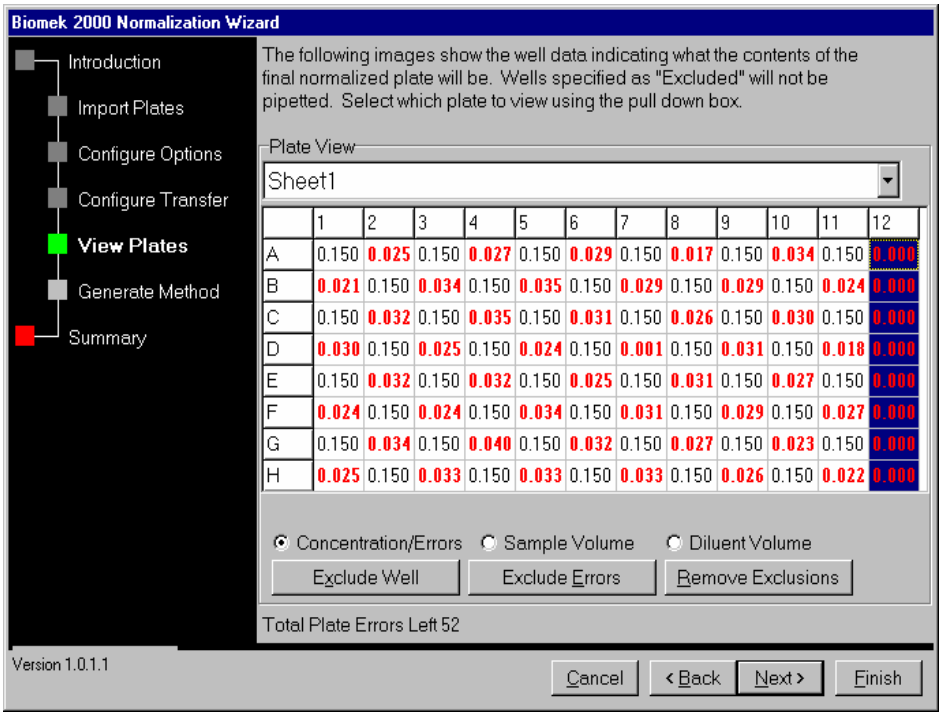


Figure 9. Setting mix values.

5.5.6 The View Plates dialog screen (Figure 9) allows the user to exclude samples or empty wells that will not be used in the procedure. Highlight the wells that need to be excluded and click on the "Exclude Well" button. The sample volume and diluent volume used can also be viewed by clicking on the circle next to the Sample Volume or Diluent Volume designation; select "Next" to proceed.

NOTE: Samples that fall below the specified range (0.15 to 0.20 ng/µL) will be shown in red.



	1	2	3	4	5	6	7	8	9	10	11	12
A	0.150	0.025	0.150	0.027	0.150	0.029	0.150	0.017	0.150	0.034	0.150	0.000
B	0.021	0.150	0.034	0.150	0.035	0.150	0.029	0.150	0.029	0.150	0.024	0.000
C	0.150	0.032	0.150	0.035	0.150	0.031	0.150	0.026	0.150	0.030	0.150	0.000
D	0.030	0.150	0.025	0.150	0.024	0.150	0.001	0.150	0.031	0.150	0.018	0.000
E	0.150	0.032	0.150	0.032	0.150	0.025	0.150	0.031	0.150	0.027	0.150	0.000
F	0.024	0.150	0.024	0.150	0.034	0.150	0.031	0.150	0.029	0.150	0.027	0.000
G	0.150	0.034	0.150	0.040	0.150	0.032	0.150	0.027	0.150	0.023	0.150	0.000
H	0.025	0.150	0.033	0.150	0.033	0.150	0.033	0.150	0.026	0.150	0.022	0.000

Figure 10. View Plates.

5.5.7 If the desired wells have been excluded from the procedure then answer "Yes" to the next prompt (Figure 11), if changes need to be made select "No" and the View Plates box in 5.5.6 reappears.

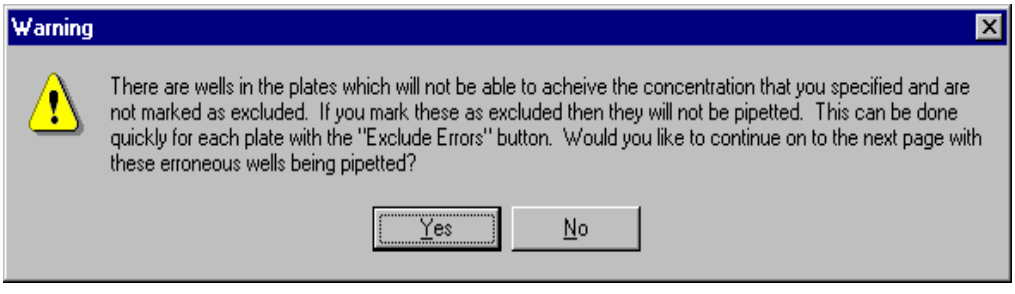


Figure 11. Exclude errors prompt

5.5.8 Verify that the caps from the strip tubes, containing the samples to be analyzed, have been removed, then select "OK" from the Warning dialog box (Figure 12).

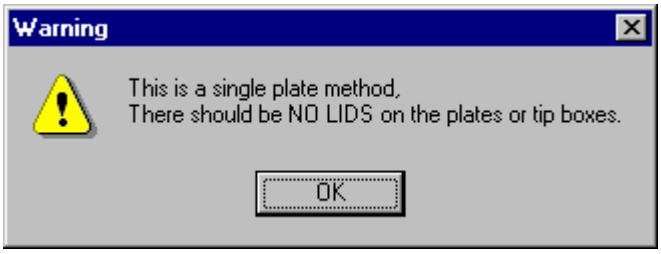


Figure 12. Warning dialog box.

5.5.9 The Generate Method dialog screen will appear (Figure 13). The Method name will have the date and time that the normalization procedure was created (i.e., Norm04/19/2004 15:48). **DO NOT CHANGE THE METHOD NAME.** Click on the 'Generate Method' button to create the new normalization method to be created. This creates the customized dilution method which can be accessed in the Lab Book that is currently open. Delete the specific normalization method generated after completion, using the delete function under Edit in the Bioworks folder.

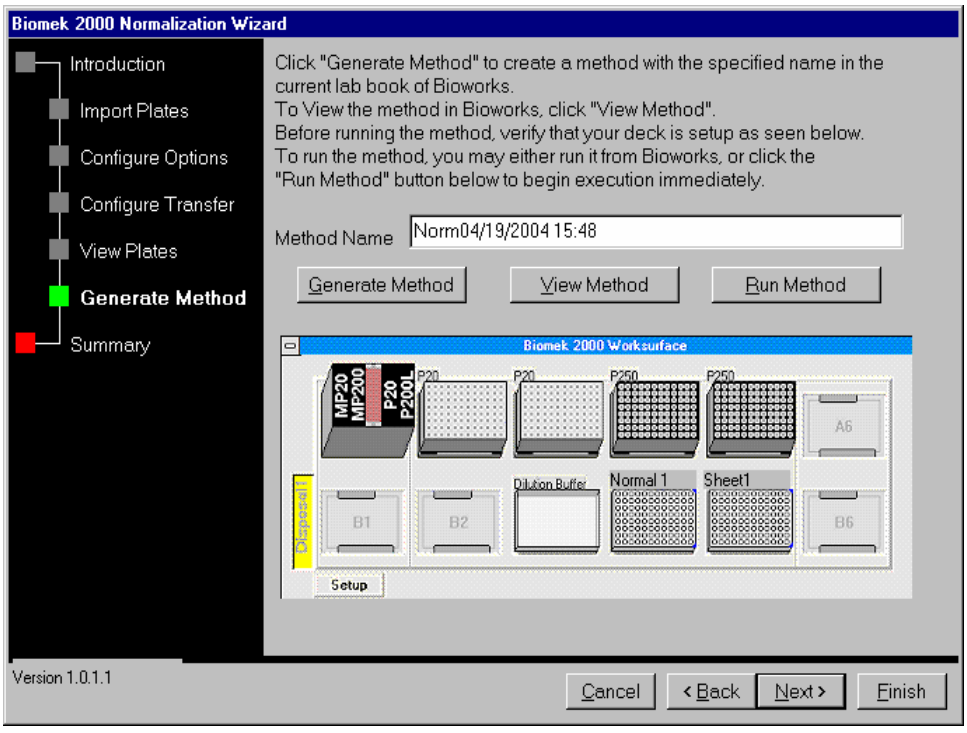


Figure 13. Generate Method

5.5.10 Click on the "Next" button to proceed.

5.5.11 Place a check the "Open in Excel" box on the Summary dialog screen, then click on the "Save Plate Maps" button. Select "Finish" to proceed.

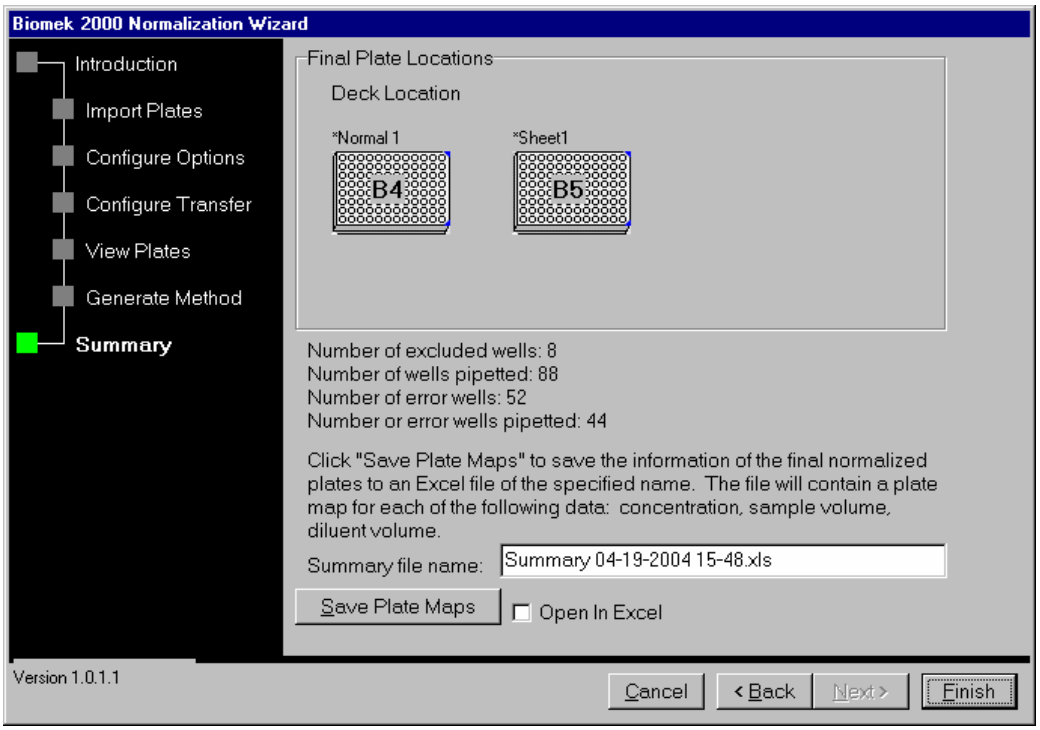


Figure 14. Summary dialog box

5.5.12 If the Excel spreadsheet containing the Plate Map information doesn't immediately come up, it can be accessed using the Start Menu. Scroll up to Documents and when the side window listing the Recent Documents opens, select the file of interest. The file will be named with the current date as shown in Figure 14. The date and operator's initials will be typed into the top header of the summary sheet (Figure 15).

NOTE: If the 'Open In Excel' was not selected then the summary of the plate maps can be retrieved by going to the C Drive then the Bioworks folder, the QNdata folder and then the summary folder. The filename will be the date and time that the summary was created (i.e. Summary 04-19-2004 15-48.xls).

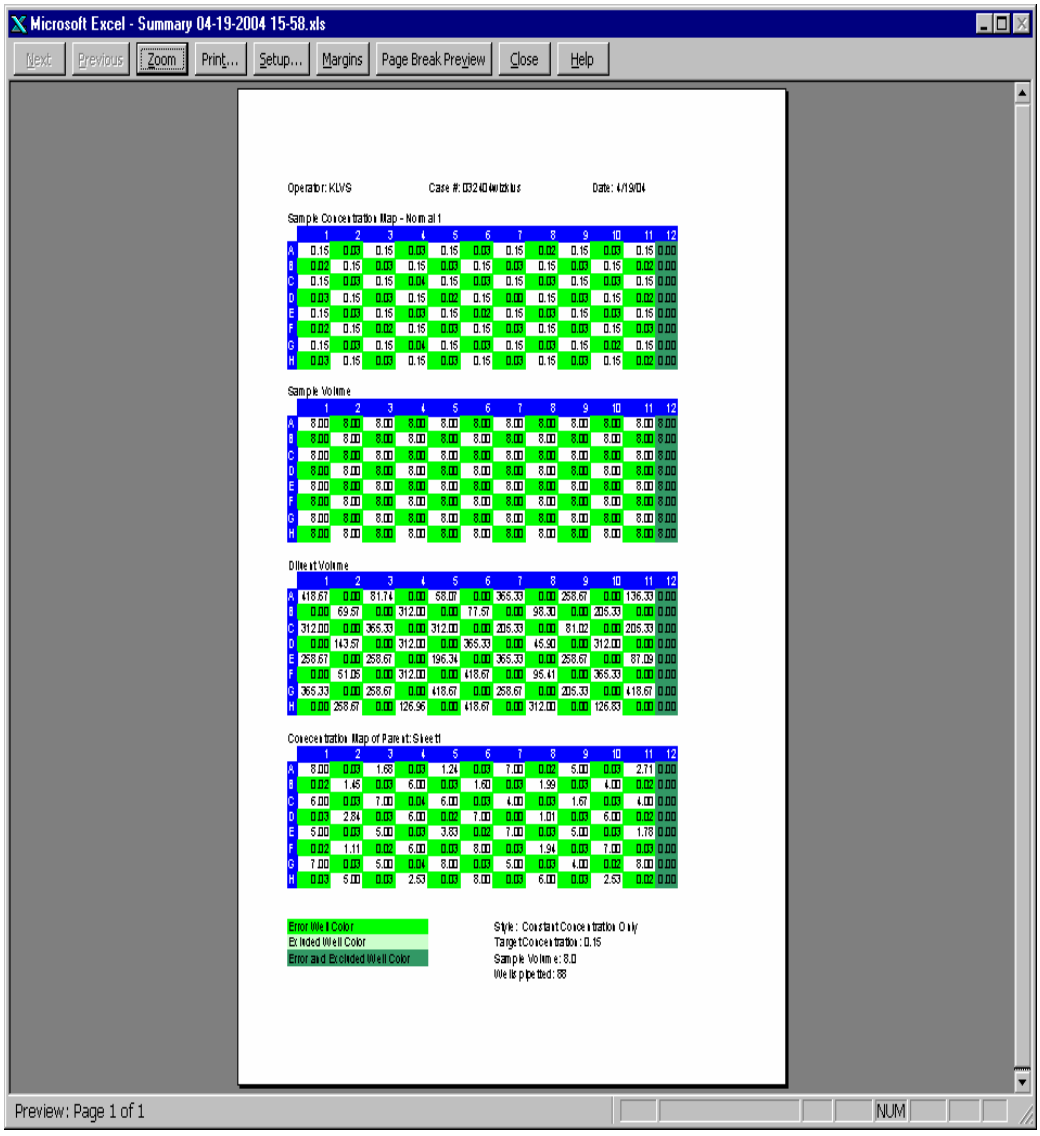


Figure 15. Summary plate map

5.5.13 Print the Plate Map sheet, then exit the Normalization Wizard program. Double click on the Edit button in the BioWorks folder to open the method list under the edit function. Locate and select the Nomalization method generated (e.g., Norm04/19/2004 15:58) as represented in the Figure 16.

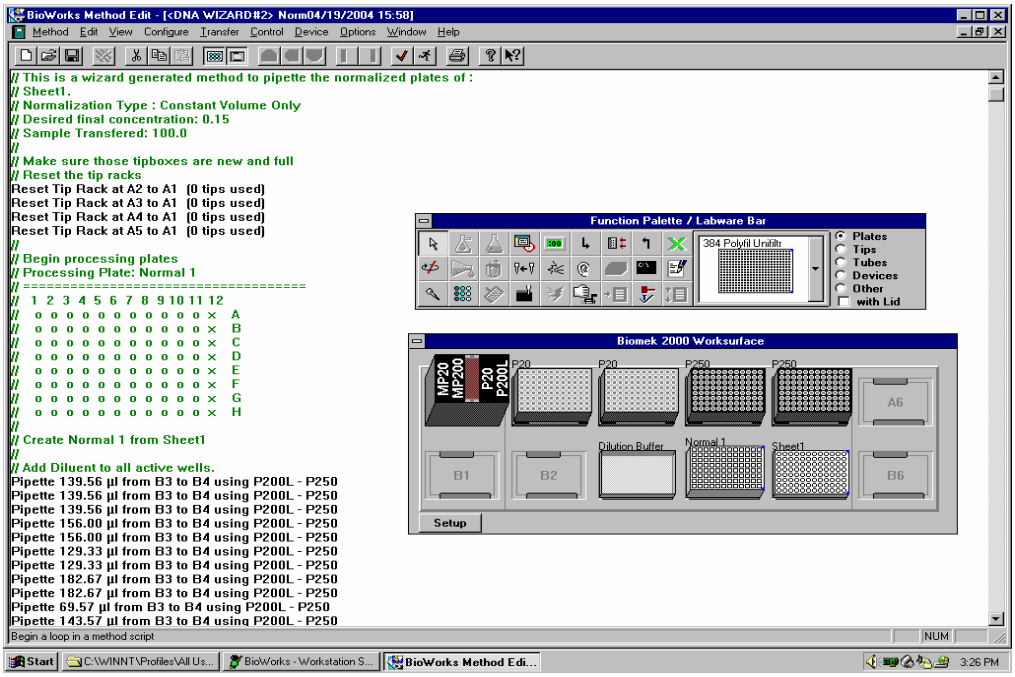


Figure 16. Normalization method

5.5.14 Use the deck layout (e.g., in Figure 16) as a guide to place the labware onto the robot deck.

5.5.14.1 Verify that the tools located in deck position A1 are in the correct order since it is different from the set up for Aluquant® procedure. Additionally, the P20 tool is used during the Normalization Wizard and the PCR amplification set up methods.

5.5.15 Place P20 barrier tips in deck position A2.

NOTE: The deck layout will have a second box of P20 barrier tips in deck position A3. The second box of tips is used in the PCR amplification set up. It will also have a box of P250 barrier tips at deck position A5 also used in the PCR amplification setup.

5.5.16 Place a box of P250 tips with NO barrier in deck position A4. These tips are utilized for pipetting the diluent (sterile water) only and lack of a filter barrier allows the tool to pipette larger volumes of the diluent.

5.5.17 Place a Full Modular Reservoir with approximately 10 mL of Type 1 sterile water in deck position B3.

5.5.18 Place a 96-well normalization plate (1.1 mL Innovative Microplate) with well A1 in the upper left corner at deck position B4. This plate has a set maximum volume of 1.145 mL, which is less than the actual volume of the plate wells, but allows for displacement during mix steps.

5.5.19 In deck position B5, place the black thermal cycler rack containing the strip tubes of extracted DNA samples. The tubes must be in the same orientation and position as indicated on the BioMek/AluQuant loading sheet.

5.5.20 When all the tools and labware are in the proper position, click on the running man button on the menu bar to initiate the method.

5.6 SETTING UP THE PCR AMPLIFICATION REACTION

5.6.1 Once the Normalization procedure is completed, close the window but remain in the Normalization Lab Book and open the 'PCR AMP SETUP' method (Figure 17). The PCR AMP SETUP method requires some customization based on the plate of samples that are to be amplified.

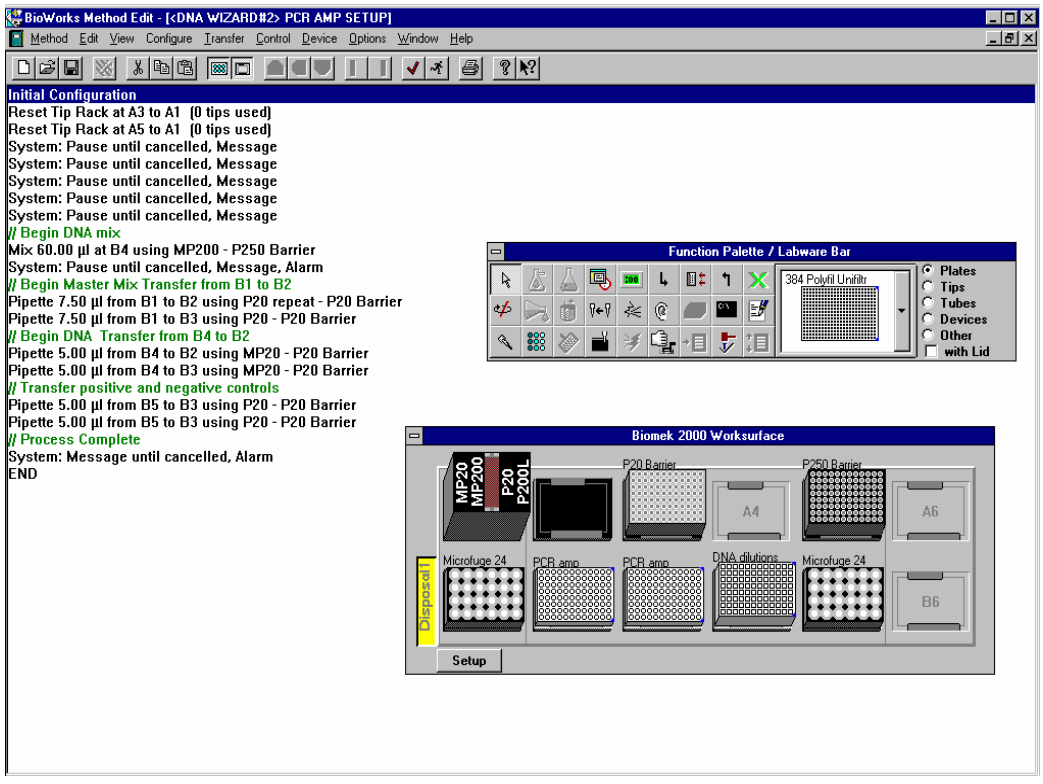


Figure 17. PCR Amp Setup method.

5.6.1.1 Use the deck layout in Figure 17 as a guide to set up the labware. Remove the used box of P20 tips at deck position A2 and the barrier free P250 tips at deck position A4. The box of barrier free P250 tips does not need to be discarded. Since only one tip is used for each Normalization Wizard setup, that tip can be removed manually and a clean, unused tip moved into the vacant position in the top left of the tip box, A1.

NOTE: If a clean tip is not placed in the A row of the first column, the robot will automatically search the next row for an available tip.

5.6.2 At deck positions B1 and B5 place microfuge tube racks that can hold 24-1.5 mL pre-labeled microcentrifuge tubes.

5.6.2.1 If amplifying more than 72 samples, the robot will pause and beep indicating that the used box of P20 tips be discarded and replaced with a new, unused box of P20 tips.

5.6.2.2 Verify that a box of P250 barrier tips has been placed in deck position A5.

5.6.2.3 Prepare the PCR master mix just prior to dispensation into the PCR amplification tubes. This can be done while the robot is mixing the diluted DNA samples in the Normalization plate.

5.6.2.3.1 If prepared prior to initiating the method, vigorously mix the PCR master mix just prior to the robot pipetting the master mix into the PCR amplification tubes.

5.6.2.3.2 Count the number of samples that need to be amplified and add an additional 20 if greater than 56 samples will be amplified. Add an additional 12 if less than 56 samples will be amplified. Include master mix volume for the positive and negative controls. Blank wells, not containing plate controls and/or reagent blanks, should NOT be included in the sample count since these wells will be excluded from the addition of PCR master mix.

5.6.2.3.3 Multiples of the positive and negative controls will be prepared for each set of samples to be amplified. For a 44 or less sample run, prepare enough master mix for two positive controls and two negative controls. For a 45 or greater sample run, prepare enough master mix for four positive controls and four negative controls.

5.6.2.4 Double click on the "Mix 60 µL at B4….." line to open the window shown in Figure 18. On the Mix at Location screen, highlight the number of columns that need to be mixed for the diluted DNA samples in the Normalization plate. Since the MP200 tool is used, all wells in those columns will be mixed, including the empty ones. Click OK when finished.

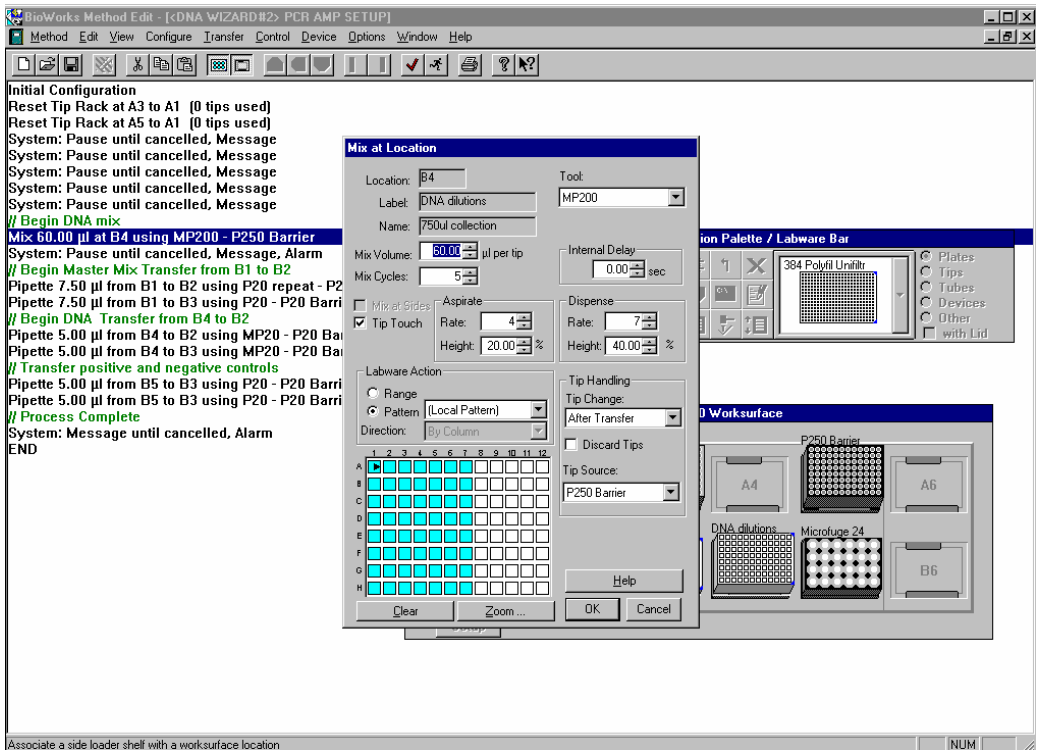


Figure 18. Designating columns of diluted DNA samples to mix.

5.6.2.5 For the PCR master mix dispensing steps, the 0.2 mL attached cap amplification tubes must be pre-labeled. Computer generated labels will be placed on all 0.2 mL amplification tubes by the reporting examiner or their designee prior to the PCR amplification set up.

5.6.2.6 Since the 0.2 mL PCR tubes that will be used have attached caps, the pattern of samples will be extended so that each column of PCR tubes is separated by a blank column in order to accommodate the caps (Figure 19A, represents samples 1 - 48 and Figure 19B, represents samples 49 – 88, plus positive and negative amplification controls or just the positive and negative amplification controls if 48 or less samples have been amplified). The pattern of the samples will be maintained. The 11th column in second PCR plate, will be designated for the set up of the positive and negative controls. NOTE: PCR tubes **SHOULD NOT** be placed into wells that **DO NOT** contain a sample/reagent blank/plate blank.

NOTE: The uncapped PCR tubes should be labeled ahead of time and placed into the black PCR support base. The uncapped tubes should be covered with a piece of parafilm to prevent anything from falling into the tubes while stored in the support base off of the deck.

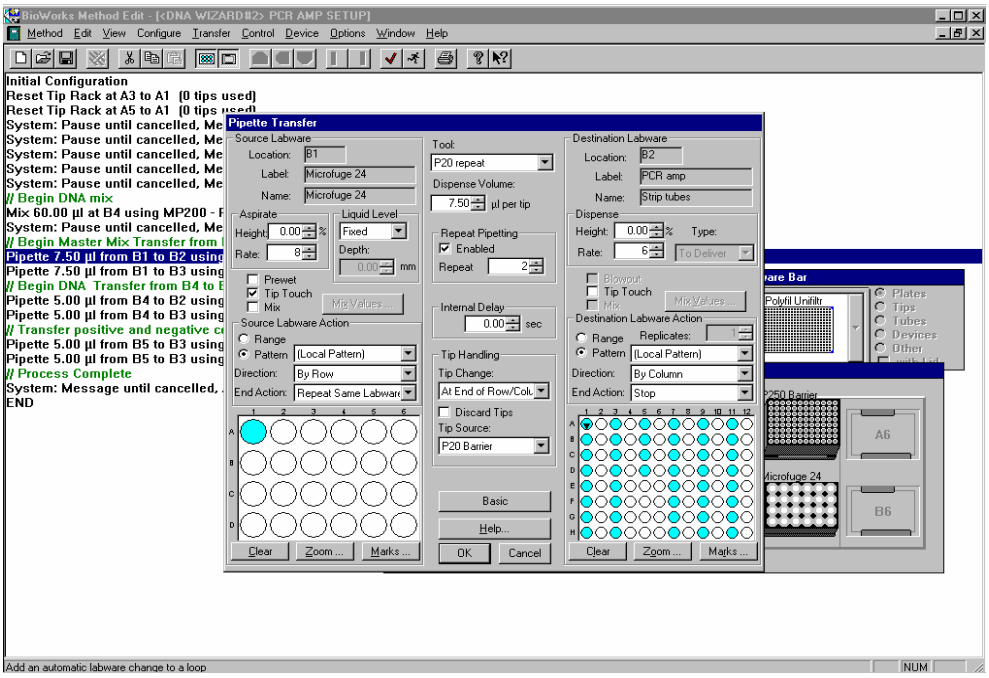


Figure 19A. Window for pipetting from B1 to B2

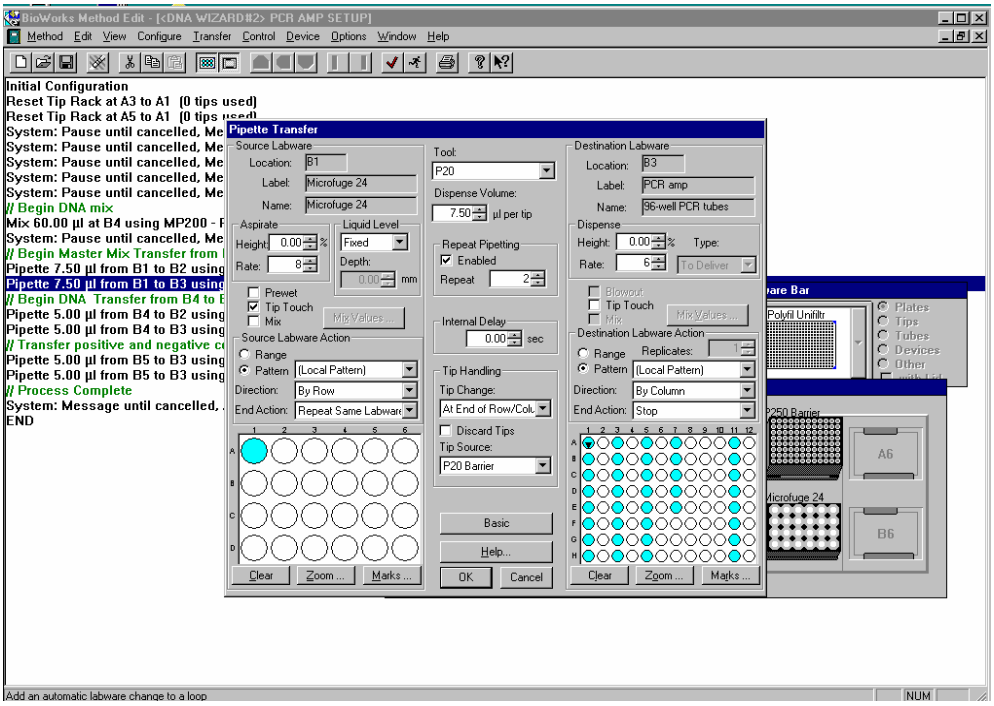


Figure 19B. Window for pipetting from B1 to B3

5.6.2.7 Double click on the black line "Pipette 7.5 µL from B1 to B2 using P20 repeat – P20 barrier" to open the window shown in Figure 19A. This line is just below the green

line stating "Begin Master Mix Transfer". Double click on the "Pipette 7.5 µL from B1 to B3…." to open the window shown in Figure 19B.

5.6.2.8 The Pipette Transfer windows will open as shown in Figures 19A and 19B. The window displays the settings for the aspiration from the 1.5 mL microfuge tube containing the PCR master mix shown in plate position A1 on the left side of the window, known as the "Source Labware". The window also displays the dispense settings of the master mix into the PCR strip tubes for the amplification on the right side of the window, known as the "Destination Labware".

5.6.2.9 Using the BioMek/AluQuant loading worksheet as a guide for the samples and reagent blanks, highlight the wells on the right, lower portion of the Pipette Transfer window (i.e., 96 well grid) which contain samples and reagent blanks. In addition, highlight the wells in the 11th column of the second plate where the positive and negative controls are to be pipetted. The BioMek® robot will skip the non-highlighted tubes/wells. Click OK for the settings in the windows shown in Figures 19A and 19B when finished.

NOTE: This specific well designation needs only to be performed at the PCR master mix transfer step and when the specific tubes are designated for dispensing positive and negative controls. A single channel pipette tool will be used to dispense the master mix into the tubes/wells that have been designated. A multi-channel pipette tool is used to transfer the diluted DNA sample to the PCR tube. Only air will be transferred to wells that contain no sample/reagent blank/plate blank.

5.6.2.10 Place a 24 well Microfuge holder in deck position B1 and B5 containing white inserts that will hold 1.5 mL tubes firmly in place. Place the capped 1.5 mL centrifuge tube containing master mix in deck position A1 of the Microfuge holder at deck position B1. Place a 1.5 mL tube containing the appropriately diluted positive control (9947A) into deck position A1 of the Microfuge holder at deck position B5. Place extra diluted positive control (approximately 10 additional uLs) into the tube to insure there is a sufficient quantity. Place a 1.5 mL tube containing approximately 50 µL of the negative control (sterile Type I water) into deck position A3 of the Microfuge holder at deck position B5. The operator will be given a prompt when to uncap the lids.

5.6.3 As shown in Figures 20A and 20B, double click on the method line stating "Pipette 5 uL from B4 to B2 using MP20-P20 Barrier" that appears just below the line in green stating "Begin DNA transfer from B4 to B2". The columns of DNA to be transferred must be selected under Source labware and using the Pipette Transfer window that pops up. Under the Destination labware, select the alternating columns of PCR tubes to which the DNA needs to be transferred. The software is capable of directing the robot to function in sequential order (eg. transferring the 2nd column of normalized DNA in the Normalization plate to the 3rd column containing the PCR tubes). Normalized DNA columns 1-6 may be transferred to the first 96 well PCR support base containing 6 alternating columns of amplification tubes. Click OK when finished.

5.6.4 Pipette on the line just below the line described above (Pipette 5 uL from B4 to B3 using MP20-P20 Barrier) to transfer the remaining columns of normalized DNA also using the alternating columns of amp tubes in the Destination labware setting. Do not select the positive and negative control column. Click OK when finished.

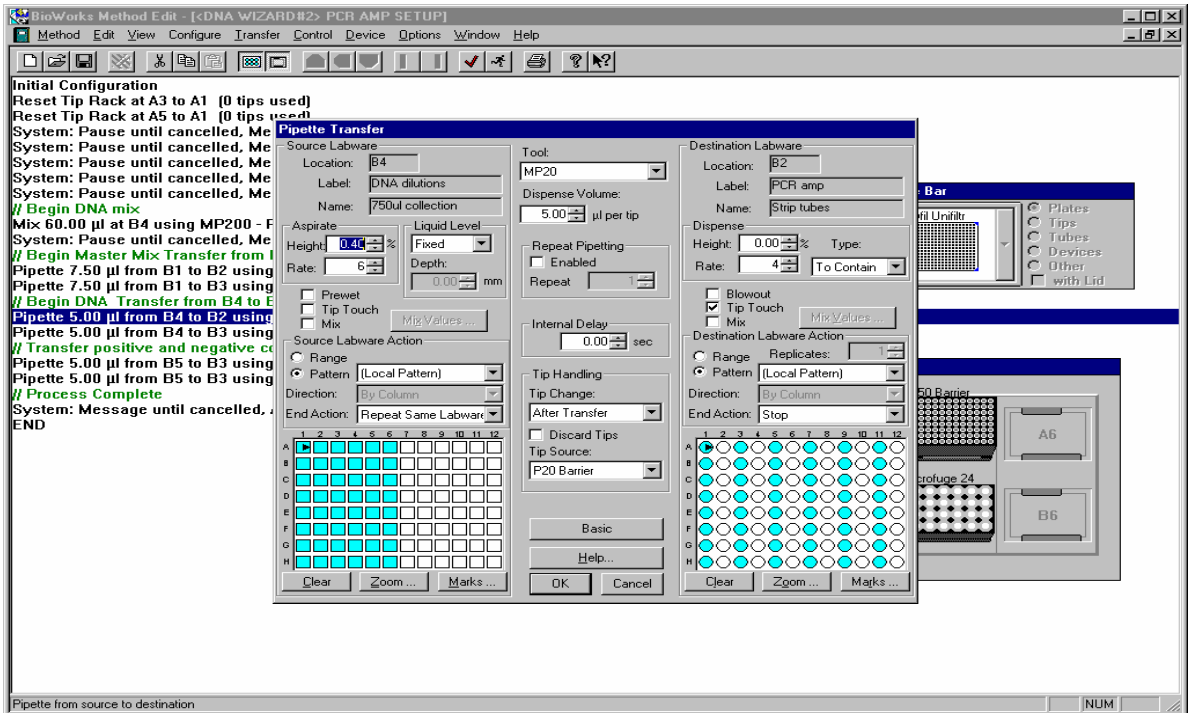


Figure 20A. Transferring the normalized DNA samples to the amplification tubes.

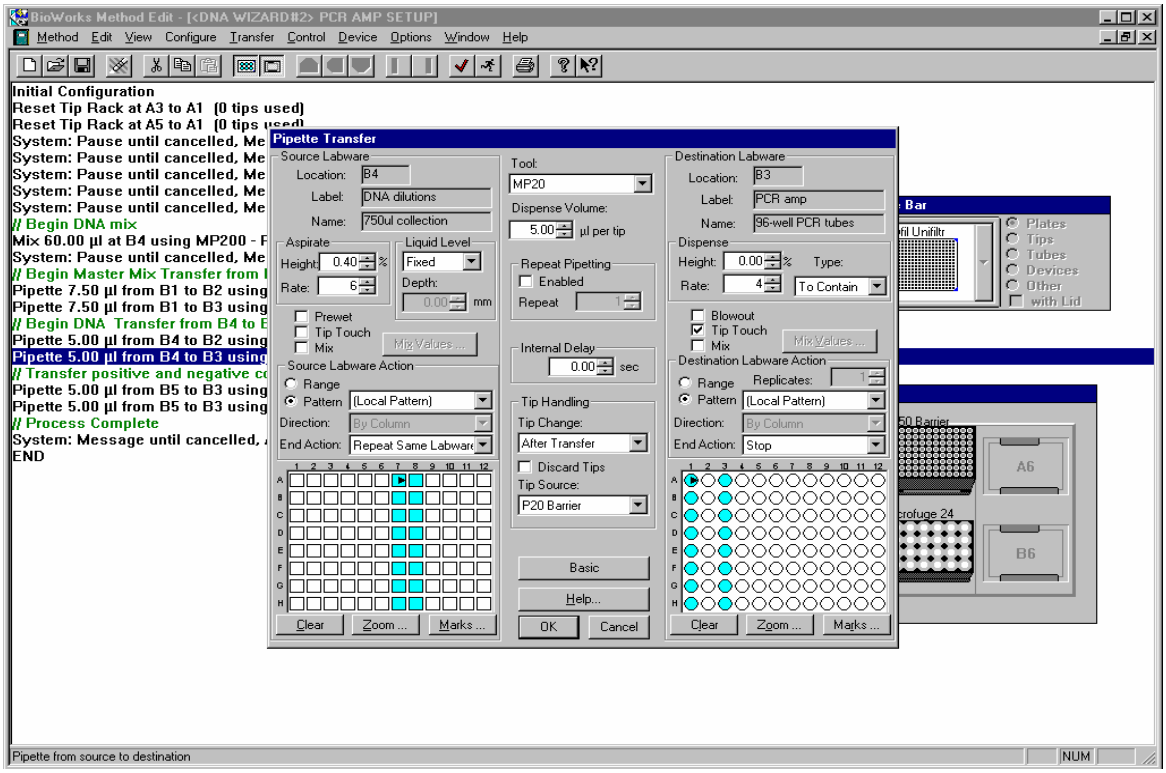


Figure 20B.

5.6.5 In order to instruct the robot into which tubes to pipette the positive control, double click on the line "Pipette 5 uL from B5 to B3 using MP20-P20 Barrier" that appears below the green line "Transfer positive and negative controls" (Figure 21). This step will only need to be customized for the Destination Labware to accommodate the number of tubes to amplify positive control. Highlight those tubes into which the positive control will be pipetted. Click OK when finished.

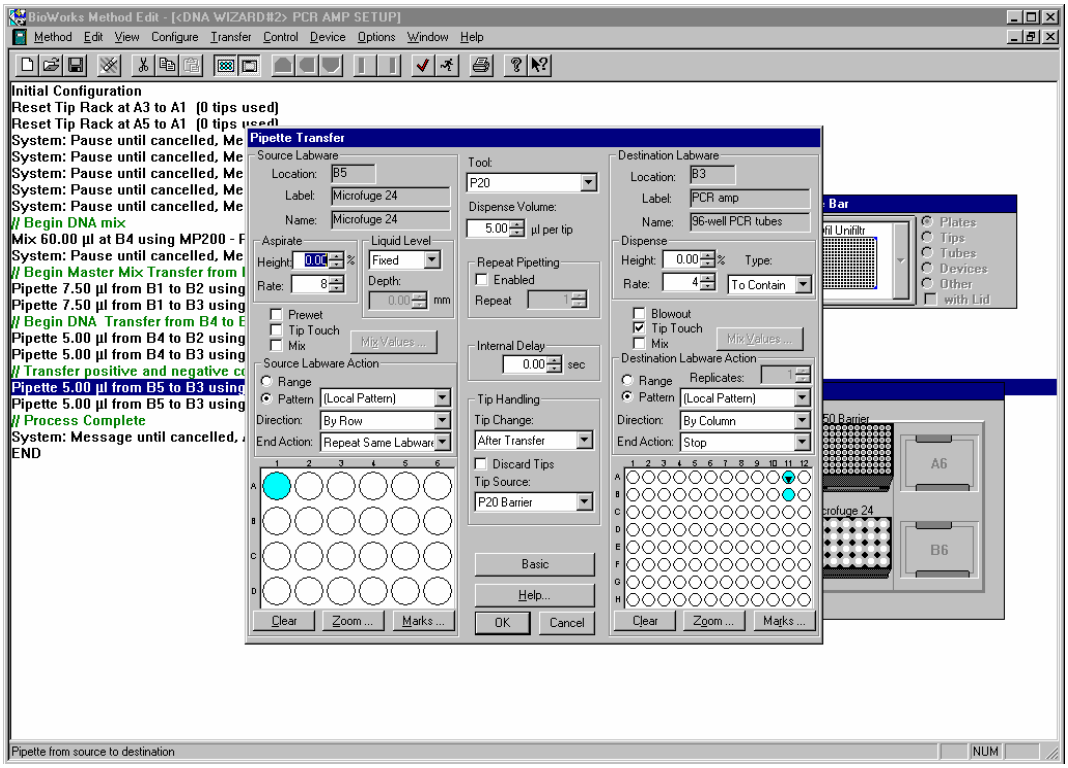


Figure 21. Transferring the positive control.

5.6.6 Double click on the line below the line described above in order to define the transfer of the negative controls (Figure 22), "Pipette 5 uL from B5 to B3 using MP20-P20 Barrier". This step will only need to be customized for the Destination Labware to accommodate the number of tubes to amplify negative control. Highlight those tubes into which the negative control will be pipetted. Click OK when finished.

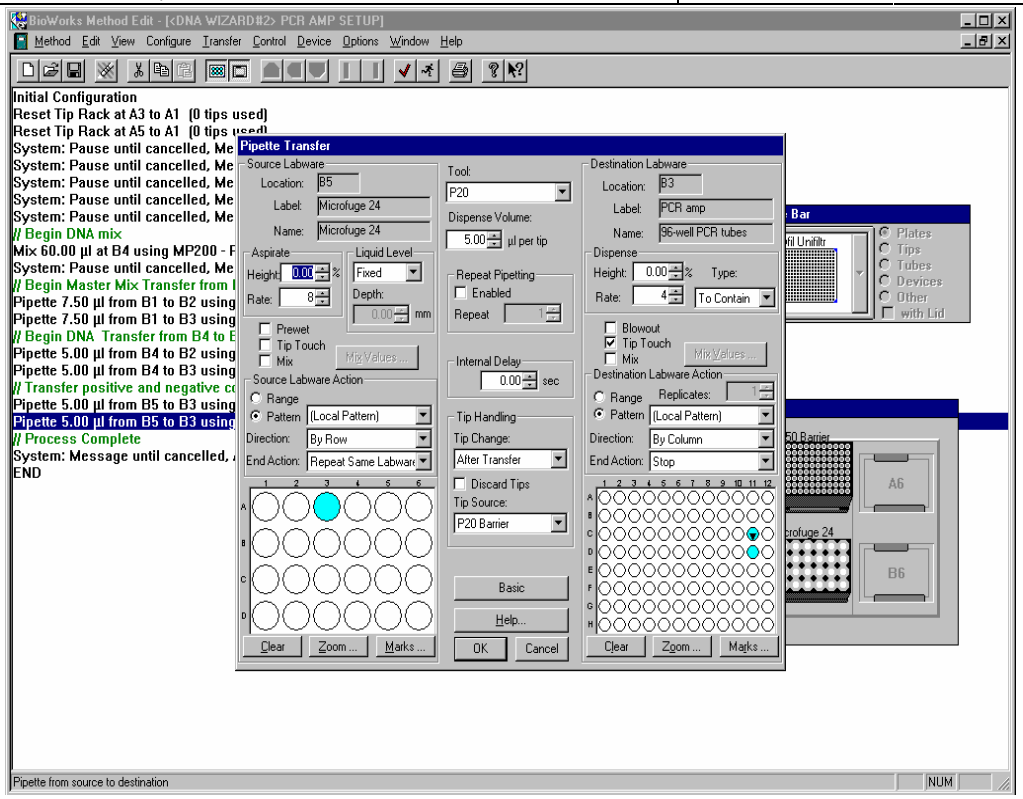


Figure 22. Transferring the negative control.

5.6.7 Once the labware is in place, click the running man button on the menu bar to initiate the method.

5.6.8 The program will initially mix all the normalized DNA samples, then once completed, the operator will be prompted to uncap the tubes containing the master mix (in position A1 of the 24 Microfuge holder at deck position B1), the positive control (in position A1 of the 24 Microfuge holder at deck position B5) and the negative control (in position A3 of the 24 Microfuge holder at deck position B5). The operator will also be prompted to place the black PCR support base onto the deck, which contains the uncapped PCR tubes that were pre-labeled by the examiner or the designee. The black PCR support bases will now serve as a thermal cycler rack for carrying the amplification tubes into the post- amplification room. Select 'OK' when this has been completed.

NOTE: Remove the parafilm from labeled PCR tubes before placing the support base(s) onto the robot deck.

5.6.9 The master mix will be aliquoted to the individual PCR amplification tubes by the robot and then DNA sample added to the PCR tubes containing the master mix. The process takes approximately 30 minutes for 88 samples.

5.6.10 Once the procedure has completed, close the attached caps of the PCR tubes containing samples and the positive and negative controls.

5.6.11 The entire rack of PCR- prepared samples is ready for transfer to the post-amplification room and for placement into the thermal cycler.

♦END